A Study of the Effects of Incubation with Certain Steroids on the Phagocytic Properties of Fibroblasts

by

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I would like to thank Dr. Klaus Bensch for his endless patience and guidance, and Miss Sandy Lesnieski for her invaluable technical assistance.

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able technical assistance.

"Lysosomes stand out in a unique fashion against all other subcellular constituents by their polymorphism and by the variety of processes both physiological and pathological, in which they are implicated. Underlying this remarkable diversity, which exists not only between different cell types, but even within single cell groups, there are a number of basic dynamic constants which can be recognized throughout the animal kingdom, from the lowest protozoon to the most specialized cells of higher vertebrates."[1]

The lysosome is a cytoplasmic organelle, contained within a single unit membrane, and characteristically staining positively for acid phosphatase and related enzymes.[2] Lysosomes average about four tenths of a micron in diameter, and are filled with various lytic enzymes which mediate the digestive and lytic functions of the cell (see table one). In fact, they can be thought of as representing an analogue of a multi-faceted digestive tract on the cellular level.[1]

Although visible with appropriate fixation and staining under the light microscope, they were not identified until 1955, when de Duve[3] separated a cell fraction, intermediate in density and size between that of mitochondria and microsomes, which appeared to contain about ten percent of all the nitrogen content and approximately forty percent of the acid phosphatase activity of the homogenate. A large

TABLE ONE[6]

SUBSTANCES FOUND WITHIN LYSOSOMES

ENZYMES	OTHER SUBSTANCES
Acid phosphatase	Phagocytin
Acid ribonuclease	Cationic "inflammatory" protein
Acid deoxyribonuclease	Mucopolysaccharides and glycoproteins
Acid protease; cathepsins	Plasminogen activator
Phosphoprotein phosphatase	Permeability-inducing protease
B-Glucuronidase	Hemolysin (or hemolysins)
B-N-acetylglucosaminidase	Unidentified basic proteins
α-mannosidase	
B-galactosidase	
α1-4 glucosidase	
Hyaluronidase	
Lysozyme	
Phospholipase, acid Lipase	
Phosphatidic acid phosphatase	
Collagenase	
Aryl sulfatases A and B	
Nonspecific esterases	

group of enzymes, operating optimally at pH 5, was also present in this preparation. Enzyme activity was increased by the addition of surface active agents such at Triton X-100 to the incubation mixture, by stirring the homogenate in a Waring blender, or by decreasing the osmotic pressure.

The investigators thought that they were dealing with a new type of cellular organelle, delimited by a single membrane, responding to osmotic shock, and containing acid hydrolase, and they called it lysosome.

Novikoff et.al., in 1956,[4] examined a pellet of this homogenate under the electron microscope, and, among the many mitochondria, discovered a few dense bodies, which were later shown histochemically, by the Gomori Technique, to contain acid phosphatase. These organelles appeared to be identical with the "peri-biliary dense bodies" Novikoff had previously described in rat liver preparations.[5]

Lysosomes now appear to be ubiquitous, and have been identified in the following tissues: liver, spleen, kidney, brain, gut, thyroid, leukocyte, macrophage, pancreas, prostate, ovary, uterus, muscle, heart, synovium, skin, spinal ganglion, bladder, etc.[6]

PHAGOCYTOSIS AND DIGESTION

Two types of digestion are carried on within the cell: 1) autophagic digestion, in which endogenous elements are broken down, secondary to fasting, anoxia, or active catabolism,[7,8,9]

and 2) endocytic digestion, in which material from outside the cell is brought within the cell and digested. These materials range from complete cells and particles to small molecules and ultramicro fluid droplets. Lysosomes are involved in both of these types of digestion. Because of the above variations in digestive function, and their presence in cells carrying on many different physiological and pathological functions, they are highly pleomorphic, probably more so than nuclei, mitochondria, or any other subcellular structure.[6,9]

Lysosomes which contain no material to be digested, are called storage granules.[6] Ingestion of a particle to be digested takes place by invagination of the cell membrane, with eventual renewal of the membrane as ingestion is completed. Thus, the engulfed material lies within a pouch composed of the inverted cell membrane.[10] This is called the endocytic vacuole, phagosome, or heterophagosome. Phagosomes containing endogenous material to be digested are called autophagic vacuoles. Lysosomes which have already carried out a digestive function, frequently appear filled with debris, usually lipid in nature, and are then called residual bodies. Several layers of debris may be found in heterolysosomes which have undergone phagocytosis and digestion a number of times.[6] Thus, heterolysosomes which had already digested colloidal iron, would later digest a second feeding of DNA-protein-colloidal gold coacervates.[11]

Briefly described below is a review of the literature dealing with the problem of intracellular phagocytosis and digestion.

Observations from motion picture[12] and electron microscope[13,14] studies indicate that fusion occurs between the membrane delimiting the heterophagosome, and that surrounding the storage granules, with a resultant discharge of these granules directly into the heterophagosome, forming the phagolysosome, or heterolysosome. Of course, depending upon the relative sizes of the two particles, this may appear as storage granules discharging into heterophagosomes, or heterophagosomes shedding their contents into storage granules, or heterophagosomes and storage granules sharing their contents.[1]

In motion picture studies of rabbit polymorphonuclear leukocytes,[12] storage granule lysis was seen early in the course of engulfment. As soon as a firm attachment between the phagocyte and particle had been made, and the enveloping process begun, granules in contact with the invaginating membrane began to rupture. It appeared that the granules ruptured into enlarging vacuoles forming around the engulfed organisms, into the space between the microorganisms and the invaginated cell membrane surrounding it.

In another study,[14] rabbit polymorphonuclear leukocytes were adhered to a glass slide, and allowed to engulf chains of group A streptococci. Control specimens showed no change

in granulation, while those exposed to the cocci showed a progressive loss of granules. Degranulation occurred within thirty minutes of phagocytosis, and was directly related to the quantity of material engulfed.

It has been pointed out that as heterophagosomes and heterolysosomes carry out multiple episodes of fusion, and since their size does not increase indefinitely, one can conclude that fission of the merged particles must also be occurring frequently.[15]

Residual bodies are often found, and because of their common presence, it appears that disruption of the residual bodies is not the usual way in which the end products of digestion are dealt with. What probably occurs, is that the lysosomal membrane becomes selectively permeable to certain small molecules, facilitating their transport into the cytoplasm, while retaining the macromolecules which are too large to be utilized there.[1]

Extrusion of digestive debris which cannot be utilized by the cell, from the residual body, to the exterior, is termed exoplasmosis.[7] Evidence for this function, however, is scanty. Merger of "peri-biliary dense bodies" with endocytosis vacuoles, and extrusion of the material from the bodies into the bile, however, has been seen.[8]

ORIGIN OF LYSOSOMES

All lysosomes found in cells may be divided into two

classifications: 1) Primary lysosomes, or those which have never engaged in any digestive activities, and 2) Secondary lysosomes, or those which are presently engaged in digestion, or have been in the past.

It has been difficult to identify particles which definitely fit the definition of a primary lysosome. Zucker and Hirsch[7] considered polymorphonuclear leukocyte granules to be primary lysosomes. This is probably true, but polymorphonuclear leukocytes are very unusual cells. Most lysosomes visible with the light microscope in phagocytic cells are probably secondary lysosomes. Primary lysosomes must either be very small in number or else in size.[1]

Just as examples of pure primary lysosomes remain difficult to identify, a clear elucidation of their formation, too, is yet to be presented.

Novikoff[4,9,16] felt that digestive vacuoles containing acid hydrolases seemed to be formed by the golgi apparatus, in conjunction with areas of the endoplasmic reticulum which were near by. In addition, the golgi apparatus, without the endoplasmic reticulum, might produce digestive vacuoles with acid hydrolases, in the form of "dense bodies" and autophagic vacuoles. In support of this concept is the finding that the golgi apparatus may stain positively for acid phosphatases.[6]

However, against this formulation, is the fact that

some workers[2,17,18] in investigations of unicellular organisms, have implicated the rough endoplasmic reticulum in the formation of digestive vacuoles.

Brandes,[19] felt that in cells containing a preponderence of smooth endoplasmic reticulum, the golgi apparatus might be involved in primary lysosome formation, while not in those cells containing mostly rough endoplasmic reticulum.

Obviously, there is still much confusion and conflicting thought regarding the origin of the primary lysosome, and the actual process of formation may not yet have been touched upon.

PROPERTIES OF THE LYSOSOME MEMBRANE

The contents of the lysosome are surrounded by a lipoprotein membrane, thought to consist of a double mono-layer of phospholipids joined by their non-polar groups, and projecting their polar groups outward, as in the cell membrane. Onto the outer polar groups are attached various protein and carbohydrate groups which give the membrane its specific properties.[20] This membrane appears to be the same as that of erythrocytes. Thus many of the membrane active drugs, vitamins, toxins, etc. which have been demonstrated to labilize the lysosomal membrane (see table 2), are also hemolytic agents.[21,22]

Most fat-soluble steroids and hormones which exert effects on the lysosome membrane are active at approximately

TABLE TWO[6]

MEMBRANE ACTIVE SUBSTANCES

IN VITRO	IN VIVO
Labilization:	
Hypotonic conditions	Vitamin A
Freezing and thawing	Endotoxin
Low pH	Streptolysins O and S
Nonionic detergents	U-V radiation
Blendorization	X-radiation
Digitonin	2,4-dinitrophenol
U-V radiation	Antigen-antibody reactions
X-radiation	High oxygen excess
Cations (5 millimolar)	Icterogenin
Phospholipase	Ischaemia
Proteases	Anoxia
Staphylococcal hemolysin	Shock
Streptolysins O and S	Prophase of mitosis
Progesterone and testosterone	Endocytosis
DOC	Metamorphosis and tissue resorption
Diethylstilbesterol	Virus infection
Etiocholanolone and 5B-steroids	Starvation
5B-H bile acids	Thyrotropin in thyroid
Icterogenin	
Cantharidin	
Vitamin A	
Cysteine	
Lysolecithin	
Stabilization:	
Cortisol	Cortisone and analogues
Cortisone	Chloroquine
Cholesterol	Serum factors in autoimmunity
Chloroquin	"Tolerance" to endotoxin
Antihistamines	
Serum factors in autoimmunity	
B-methasone	
Prednisone	

$5X10^{-4}$ Molar, which is far in excess of physiological or pharmacological concentrations. However, if solvents such as DMSO, which make these compounds more available to the lipid layers of the surface membranes than do water solutions, are used, concentrations of less than 10^{-6} Molar have been found to be effective.[23] Table two presents a list of the agents which have been found to labilize or stabilize the lysosome membrane, either *in vitro* or *in vivo*.

Weissmann[27] stated that neutral steroids of 5B-H configuration: pregnanalone, etiocholanolone, pregnandione, 11-ketopregnanalone, and progesterone release enzymes from lysosomes and decrease the turbidity of leukocyte lysosome suspensions. These agents were only active at concentrations greater than $2.5X10^{-4}$ Molar. Since the enzyme release was seen only at 37^{o}, with the etiocholanolone precursors: testosterone, dehydroepianrosterone, 11-desoxycortisol, and androstenedione, it is possible that metabolism is necessary. No 5 -H steroids were found to be active.

Far fewer agents have been found which appear to stabilize lysosomal membranes. Among these, are the anti-inflammatory steroids: cortisone, cortisol, prednisolone, betamethasone (as acetates, phosphates, and hemisuccinates), but not corticosterone. These appear to act both *in vitro* and in the living cell.[23] In addition, they have been shown to stabilize cells against the effects of excess vitamin A,

streptolysin O, ultra-violet light, etiocholanolone-progesterone combinations, carbon tetrachloride, and simple incubation at varying pH and at several different temperatures. Similarly, they have been shown to protect the lysosomes of living cells, organs, and animals from the labilizing effects of endotoxins, antigen-antibody reactions, excess oxygen, traumatic shock, ultra-violet light, and vitamin A intoxication.[6]

Weissmann and Thomas[24] examined the effect of pre-treatment of rabbits with cortisone on the lysosomal fraction of their livers, and found that there was a definite, but modest decrease in the release of cathepsin and B-glucuronidase which was most readily seen in samples which had been both irradiated and incubated. When the animals were challenged with endotoxin, no augmentation of enzyme release into the supernatents of incubated and irradiated fractions was seen. In fact, most activities were below control values. Free activities were also significantly lower than normal. There was a decrease in total B-glucuronidase activity in the fractions both with cortisone alone, and with cortisone plus endotoxin. There was maintenance of the same cathepsin activity in both groups, however, which was not explained.

In another study,[25] when cortisone was administered together with vitamin A, release of enzymes into the unsedimentable fraction was significantly inhibited. This effect was also seen <u>in vitro</u>. Cortisol as the hemisuccinate, at

concentrations of 10^{-3} and 10^{-4}, has also been shown to retard the release of hydrolases from granules by vitamin A.[23]

In another study, cortisone, cortisole acetate, and cortisol, stabilized lysosomes against the effects of incubation at acid pH. Appreciable inhibition of enzyme release has also been shown with rabbit liver lysosomes incubated with cortisone for thirty minutes at 37° and then exposed to etiocholanolone.[23]

Chloroquine, effective at 10^{-4} Molar when dissolved in water, and at 10^{-5} Molar in DMSO,[23] protected lysosomes in vitro against the effects of lysolecithin, progesterone, etiocholanolone, vitamin A, streptolysin S, ultra-violet irradiation, and neutral pH incubation.[26,27]

Effects of these substances on the permeability of the lysosome membrane is probably an example of their effect on the lipids found in other membrane bounded structures.[23] An effect on critical areas of a dynamic membrane such as this could account for alterations in its permeability to contained enzymes.[24]

These studies seem to present a reasonable amount of evidence in support of the hypothesis that the clinically observed anti-inflammatory action of certain steroids has its effect, at least in part, on the lysosomal membrane.[24,21]

LYSOSOMES AND DISEASE

Lysosomes may function pathologically either by inadequate,

or by inappropriate action.

Usually, deviation from normal lysosomal function is the result, rather than the cause of the basic pathological process seen in the organism.[1]

In almost anydysfunction of the cell, such as autolysis, ischaemia, shock, or inflammation, the lysosome will ultimately become involved, and thus its role in the disease process is important, both from a defensive point of view, as well as a symptomalogical one.

Lysosomes have been implicated in inflammatory diseases such as the connective tissue diseases. In rheumatoid arthritis the erosion of the cartilage matrix appears to be secondary to lysosomal enzyme release from the inflammatory cells of the invading pannus.[28] In this condition, the degradation of the cartilage protein polysaccharides is very similar to that seen experimentally induced by an excess of vitamin A.[29,30] Studies indicate that a leakage of lysosomal enzyme into the joint capsule[31,32,33,34] may be induced by the effect of the rheumatoid factor on the lysosomal membrane. Further, markedly enlarged and bizarre residual bodies have been found in the macrophage-like synovial lining cells of patients with rheumatoid arthritis.[35] Repeated injections of streptolysin S[36] into the knee joints of rabbits also produced a deformity of these joints, accompanied by true pannus formation, which, in conjunction with erosion of the underlying cartilage, also closely resembled the local lesions seen with rheumatoid arthritis in humans.

Many of the agents which appear to increase the symptomatology of the connective tissue diseases such as ultraviolet light or progesterone in systemic lupus erythematosis, or the streptococcal elaborations preceding rheumatic fever, also appear to be those substances acting to make the lysosomal membrane more easily ruptured. Further, drugs, such as cortisone and chloroquine, used to treat many rheumatic diseases, seem also to stabilize the lysosomal membrane, giving further weight to the hypothesis that changes in the lysosomal membrane are involved in the connective tissue diseases.

Lysosomes have also been indicted in the production of certain types of fevers in primates only. Etiocholanolone and other 5-B steroids, which, as noted above, have been shown to disrupt lysosomal membranes *in vitro*, but not *in vivo*, have also been shown to produce fever in man after they were injected intramuscularly.[37] The febrile effects of these agents, whether administered exogenously, or produced by the individual,[38] are abolished with the administration of cortisone or its analogues which, of course, also stabilize the lysosomal membrane.[37]

MATERIALS AND METHODS

Strain L rabbit fibroblasts of known phagocytic ability[39,11] were grown in suspension culture in 250 ml. Erlenmeyer flasks agitated on a rotary shaker at 37.5° C.[40] The culture medium was prepared from Eagle's basal medium (10X), 100 ml., $NaHCO_3$, 4.2 Grams, essential amino acids concentrate, 20 ml., with double distilled water to make one liter; plus glutamine, 300 mg., sodium pyruvate, 110 mg., vitamin concentrate (100X), 10 ml., horse serum, 100-120 ml., penicillin, 100 units, and 50% dextrose solution, 2.0 ml. 70 ml. of this medium was included in each flask. The pH of the medium was adjusted to 7.3 with CO_2, using a phenol red indicator (included in the medium).

Cell density was evaluated using a standard hemocytometer, and, at a density of 1.2×10^6 per ml., the cells were transferred to fresh medium. The generation time of the cultures was 22-26 hours. The cells were transferred by first placing them in sterile centrifuge tubes and centrifuging for 1 1/2 minutes. The clumped cells were then sucked into a sterile pippette containing 3 ml. of fresh medium, to resuspend them, and then placed into a sterile 250 ml. Erlenmeyer flask. To this was added 70 ml. of fresh medium with an initial concentration of 150,000 cells per ml. 8%CO_2 in air was bubbled into the flask through a sterile glass tube for two-five minutes to bring the pH to 7.3, and the cells were then replaced in the incubator.

PARTICLES

The colloidal gold-DNA particles for the cells to ingest were prepared according to the work of Bensch and King:[41,11] Dilute, acqueous solutions of herring sperm DNA (0.1%) were added to approximately equal volumes of 0.1% gelatin in colloidal gold (Colloidal Gold Reagent-Harleco). This was performed in a water bath at 50° C., at a pH of 4. After suitably sized particles had formed, they were stabilized by exposure to 0.1-0.2% glutaraldehyde. The particles were then dialyzed for 72 hours against normal saline. Particle size and density was evaluated under the light microscope at a magnification of 1200.

STEROIDS

1.44 mg. of hydrocortisone acetate (molecular weight-404.51) was added to .275 ml. of propylene glycol, forming what appeared to be a cloudy suspension. 1.44 mg. of the hydrocortisone was also added to 0.275 ml. of double distilled water, forming a similarly cloudy suspension. Propylene glycol was used because it was hoped that the hydrocortisone would be more soluble in it than in water. The water solution was used in addition in case of adverse effects from the propylene glycol on the cells.

Dr. Morris Dillard, Department of Medicine, prepared a sample of pure crystalline etiocholanolone (molecular weight-290.43), which he dissolved in 40 ml. of nutrient

medium. The concentration of etiocholanolone in this solution was approximately 10^{-4} Molar, as measured by extraction methods with chloroform and quantitative analysis with the Zimmerman Colorimetric Reagent.

PHAGOCYTOSIS EXPERIMENT

Four sterile 250 ml. Erlenmeyer flasks were labelled A,B,C, and D, respectively. To A, was added 40 ml. of nutrient medium containing etiocholanolone at a concentration of 10^{-4} Molar, as above. To B, was added 40 ml. of nutrient medium to which nothing had been added. To C, was added 40 ml. of nutrient medium to which the hydrocortisone-water suspension had been added, forming a hydrocortisone concentration of 10^{-4} Molar in the medium. To D, was added 40 ml. of nutrient medium containing the hydrocortisone-propylene glycol suspension, forming a hydrocortisone concentration of 10^{-4} Molar in the medium. Each flask was inoculated with cells from stock cultures giving a final concentration of approximately 7.5×10^5 cells per ml. in the logarithmal phase of growth. The four flasks were then agitated on a rotary shaker for one hour at 37.5° C. After one hour of incubation, 4 ml. of the colloidal gold-DNA particles were added to each of the flasks, and they were replaced on the rotary shaker at 37.5° C. After two additional hours of incubation, the experiment was terminated by fixation and harvesting of the cells.

ELECTRON MICROSCOPY

A solution of 2% glutaraldehyde in 0.05 Molar cacodylate buffer[11,42,43] pH 7.2 in equal volume to that of the nutrient medium (40 ml.) was added to each flask. The flasks were then shaken once a minute. After ten minutes, the cells were centrifuged, the clumps cut into pieces $< 1mm^3$ in size, and covered with 10 ml. of the same fixative, and refrigerated for 1-1 1/2 hours. The buffer was then poured off, and the cells covered with 10 ml. of an isotonic wash solution of sucrose with 0.05 Molar cacodylate buffer at a pH of 7.2.[11] The cells were then refrigerated for 24 hours or more, covered with this buffer-wash solution. Next, the cells were post-fixed with an osmium tetroxide fixative.[44,45] As much of the buffer solution was removed from the cells as possible, and was replaced by 3-5 ml. of the fixative, making certain that all the tissue was covered. The cells were then fixed at 4° C for 1 1/2 hours. Following this, the cells were dehydrated by being immersed in progressively higher concentrations of alcohol up to 100%, followed by propylene oxide, and finally a 50% propylene oxide-50% maraglas[46] solution.

After dehydration, single $1mm^3$ pieces of tissue were placed into labelled polyethylene embedding capsules which had been filled with maraglas. The specimens were allowed to sink to the bottom of the capsule, and the maraglas allowed to cure at 60° C. for 24 hours.

The blocks were then readied for sectioning. The knives for sectioning were cut from glass on an LKB Knife-maker. After trimming the blocks with a single-edged razor blade, sections were cut with the LKB Ultratome. Thick sections (1 M thick) were cut first and heat-fixed on glass slides. These were then stained with a toluidine blue stain.[47]

Following this, thin sections (less than 0.05 M thick) were cut and placed on copper grids. These sections were stained with a uranyl acetate stain[48] for fifteen minutes, followed by a lead citrate stain[49] for fifteen minutes.

After staining, the thick sections were examined with the Zeiss Phase and Photomicroscope at magnifications of up to 1200 x. Under these conditions, the DNA-colloidal gold particles and digestive vacuoles in the embedded cells were clearly visible. These were counted per section of cell along with the number of clumps that the particles formed within each cell. Photomicrographs of the cells were taken with the camera unit of the microscope (see plate 1). Each thin section was examined using the Siemens Elmiskop 1 electron microscope, and photographs were taken of suitable fields, at magnifications of up to 20,000 X.

RESULTS

In the examination of the thick sections, a quantitative evaluation of certain differences between the groups of treated cells was attempted, using the phase microscope. Between sixty and one hundred cells were examined in each of the four groups of cells. These included all cells encountered in the section being examined, except those which could not be seen clearly. Thus, the sampling was as random as possible. In each cell, the number of particles, clumps of particles, and vacuoles present in the cell were noted. The number of particles was counted in order to evaluate the amount of gross movement of particles from outside to inside the cell. The number of clumps was counted because in many instances, the particles were ingested in groups, and it appeared, therefore, that the number of clumps of particles in the cells would be a closer index of the number of endocytic vasicles which had been formed during the experimental period. Vacuoles were counted in order to evaluate the number of foci of digestion (digestive vacuoles) within each cell.

The number of particles, clumps, and vacuoles per cell for each group was subjected to statistical analysis. The results of this analysis are summarized in Table 3. In the analysis of variance performed on the figures obtained, P was found to be less than 0.001, indicating that these

TABLE THREE

95% CONFIDENCE LIMITS ON THE MEAN

	Group A	Group B	Group C	Group D
VACUOLES	3.33-4.65	3.12-4.68	1.43-2.15	2.06-3.22
PARTICLES	4.95-6.71	3.63-5.87	3.29-3.41	2.91-4.51
CLUMPS	5.39-8.67	4.59-7.51	2.68-3.84	1.92-3.56

figures were highly significant. Ninety-five percent confidence limits were computed for the mean number of particles, clumps, and vacuoles in each of the four groups (see table 3). This showed a large overlap of the confidence limits of all three parameters between Group A (etiocholanolone) and Group B (control). This indicates that there was no significant difference in the numbers of particles, clumps, and vacuoles between Group A and Group B. There was no overlap of the confidence limits of Group B and Group C (hydrocortisone dissolved in water). There was overlap of the confidence limits between two out of three of the parameters of Group B and Group D (hydrocortisone dissolved in propylene glycol). These overlaps were probably not significant according to results of the T-test applied to these figures. There was significant overlap between Group C and Group D in two out of three parameters. This indicates that there was probably a significant difference between Groups C and D and Group B, but no significant difference between Group C and Group D.

The thin sections were examined with the electron microscope in order to qualitatively evaluate the four groups of cells. All cells were noted to be healthy and without any evidence of toxicity from the elements introduced into the culture media.

All stages of digestion appeared to be present

within the cells of each group (see plates 2-3b). The DNA-colloidal gold coacervates appeared as dense black particles outside the cell. The particles were surrounded and engulfed by an invagination of the outer cell membrane, and were then found within an endocytic vacuole. Storage granules appeared to discharge their contents into the endocytic vacuoles. During digestion, the particles became cloudy gray masses containing black dots (colloidal gold), and later, lost all definition, and only the black dots could be identified. Thus, the colloidal gold could be seen accumulated within the vacuoles which had already undergone at least one episode of particle digestion. Residual bodies were found in many of the cells. Photomicrographs of cells from each group undergoing various phases of digestion are included (plates 4-9). Quantitative differences could be noted only when large amounts of cells were examined with the phase microscope.

CONCLUSION

It was shown above that lysosomes function as intracellular digestive elements, and are important in both the normal and the pathological functions of a cell. Further, certain steroids have been noted to affect the lysosome membrane, both *in vivo* and *in vitro*. In addition, these steroids also play a role in certain clinical syndromes which seem to be closely involved with lysosomal function. In this study it was attempted to investigate mechanisms by which these steroids may affect the lysosome. Thus, hydrocortisone acetate, a lysosome membrane stabilizer, and etiocholanolone, a lysosome membrane activator, were incubated with phagocytic cells in order to evaluate any changes in the processes of ingestion and digestion carried on by these cells.

On the basis of the figures obtained from this investigation, the following conclusions can be arrived at: 1) There was a significant decrease in the degree of ingestion and digestion carried out by the tissue culture cells which had been incubated with hydrocortisone, with no significant difference between those cells incubated with hydrocortisone in water, and those incubated with hydrocortisone brought into solution in propylene glycol, and 2) Incubation of tissue culture cells with etiocholanolone under the above experimental conditions apparently had no effect on the degree of ingestion and digestion carried out by these cells.

The effect of incubation with hydrocortisone on the tissue culture cells is entirely in accord with the *in vitro* and *in vivo* studies and clinical observations cited above, and appears to indicate that the anti-inflammatory steroids exert at least part of their effect by decreasing the level of digestive activity in the phagocytic cell. On the other hand, any effects which etiocholanolone may have had on the lysosome membrane of cells incubated with it in this study could not be correlated with changes in the degree of ingestion and digestion found in these cells. It is impossible to completely account for a negative result such as this, however any of the following factors may be involved in producing the final results: 1) Too low a concentration of etiocholanolone in the nutrient medium to significantly effect the cells; 2) Inability of etiocholanolone to affect lysosomes from non-primates *in vivo*; 3) Too short a pre-incubation time for cells to be affected by the etiocholanolone; 4) Etiocholanolone may have no effect on ingestion or digestion in any phagocytic cell.

In conclusion, it must be noted that these results represent only a single experimental procedure, and are, therefore, limited in their significance. It is obvious that further experimentation must be done on this problem before any definite conclusions can be reached.

PLATES

The following abbreviations will be used in labelling the plates: CG - colloidal gold particle; P - DNA-colloidal gold particle; N - nucleus; R - residual body; E - endocytic vacuole; EV - membrane of endocytic vacuole; Cm - cell membrane.

PLATE 1 Experimental cells as seen with phase microscopy. Clumps (C), particles (P), and vacuoles (V) can be seen within the cells. X1200.

Plate 2a Stage 1 in digestive process: DNA-colloidal gold particles are surrounded by the invaginated cell membrane. X20,000.

PLATE 2b Stage 2 in the digestive process: Particle appears as a discrete entity within the endocytic vacuole. Stage 3: Vesicles containing digestive enzymes (arrow) discharge their contents into an endocytic vacuole containing four DNA-colloidal gold particles. X20,000.

PLATE 3a Stage 4 in digestive process: Particle has become gray and less discrete. Stage 5: Particle has lost all definition and only the colloidal gold can be identified. Stage 6: Residual body containing colloidal gold. X20,000.

Plate 3b Stage 6 in the digestive process: Two residual bodies are seen containing colloidal gold (arrows). X22,000.

PLATE 4 Endocytic vacuole within a Group A cell, containing a large clump of undigested particles (arrow). X12,000.

PLATE 5 Endocytic vacuoles in Group A cell containing particles in stage 4 of digestion, with numerous colloidal gold particles present. X20,000.

PLATE 6 Endocytic vacuoles in a Group B cell containing several large clumps of particles (arrows), one vacuole containing well digested material. Numerous particles can be seen outside the cell. X4,000.

PLATE 7 Endocytic vacuoles in a Group B cell containing particles in late stages of digestion. Cell membrane has extended pseudopodia (arrow). X8,000.

PLATE 8 Several endocytic vacuoles in a Group C cell containing undigested particles (arrows), and one vacuole containing well digested particles. A lipid droplet (L) can be seen. X8,000.

PLATE 9 Endocytic vacuoles from a Group D cell containing recently ingested particles, some in a clump, and vacuoles in which the particles have already been digested. X10,000.

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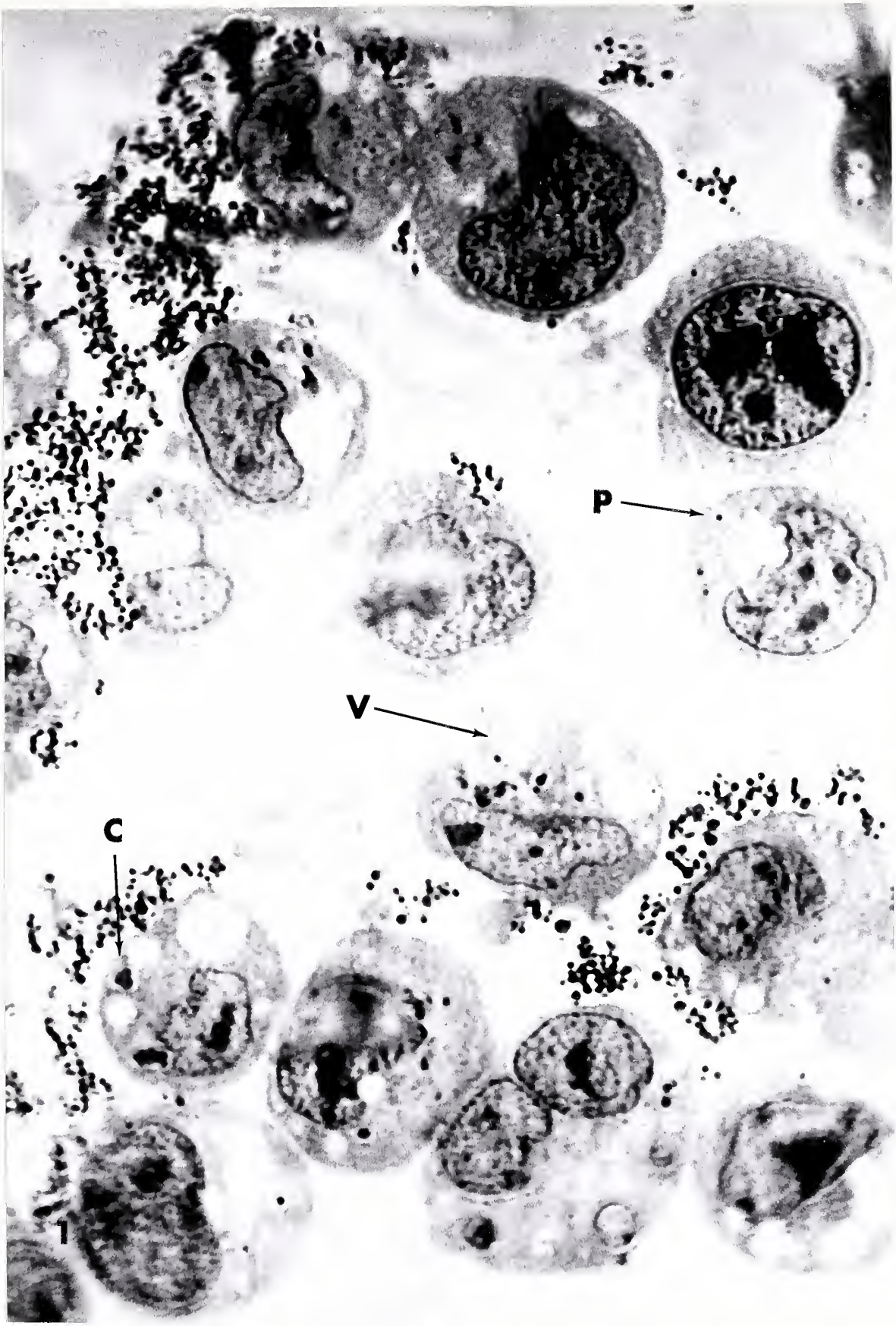
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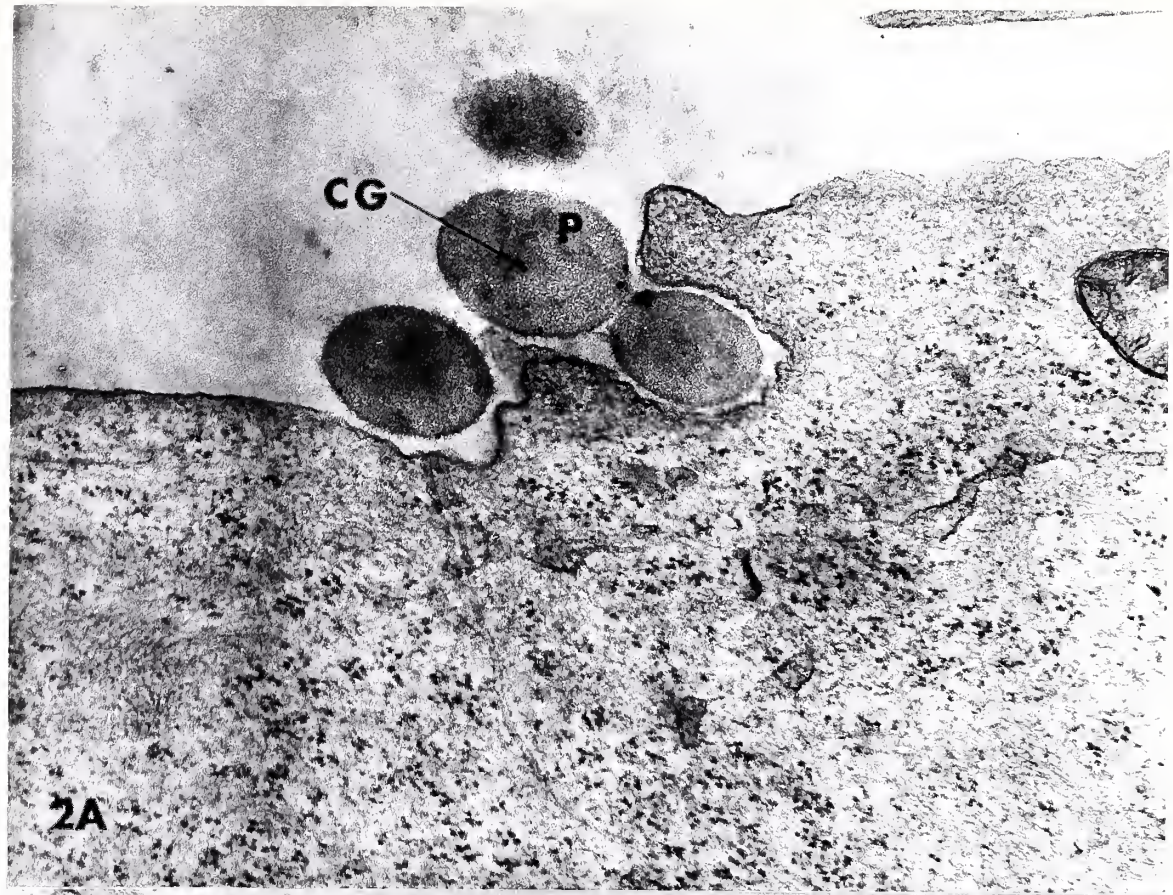
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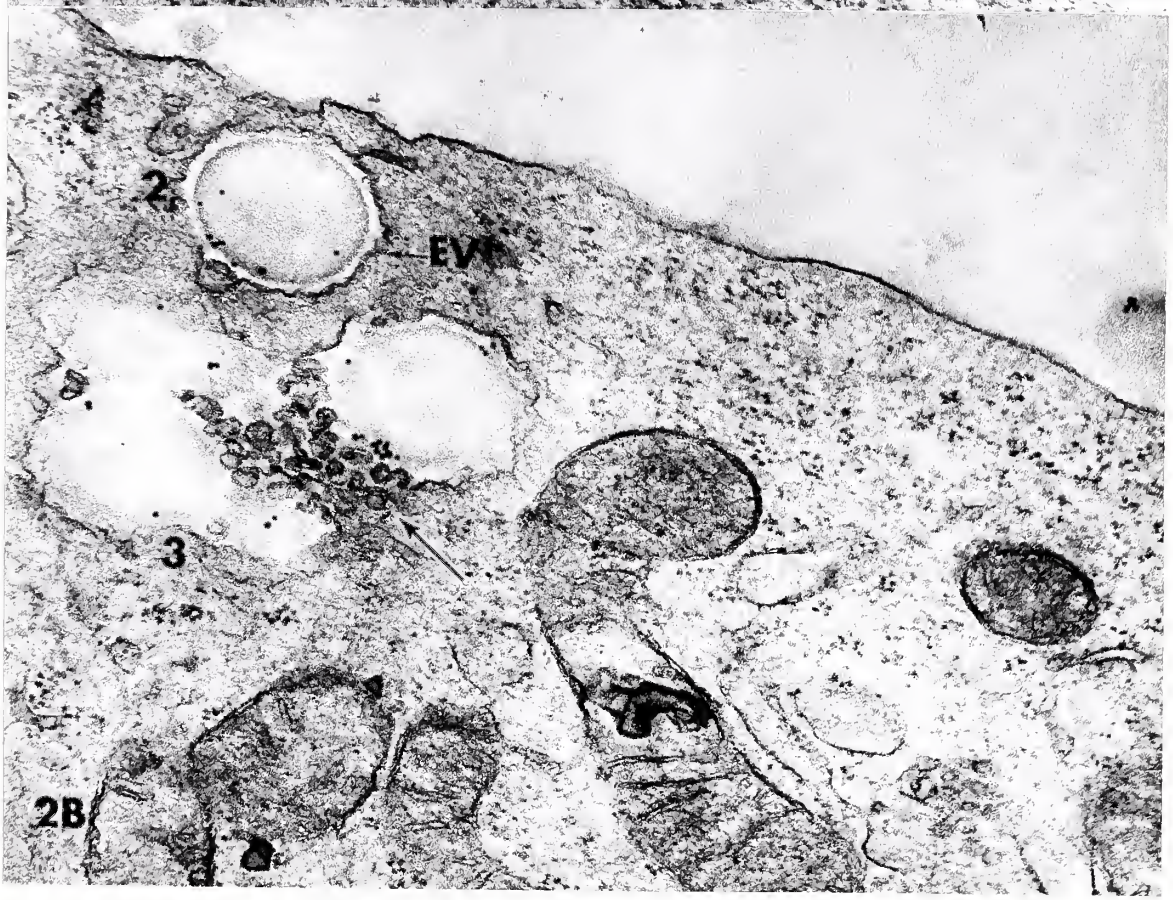
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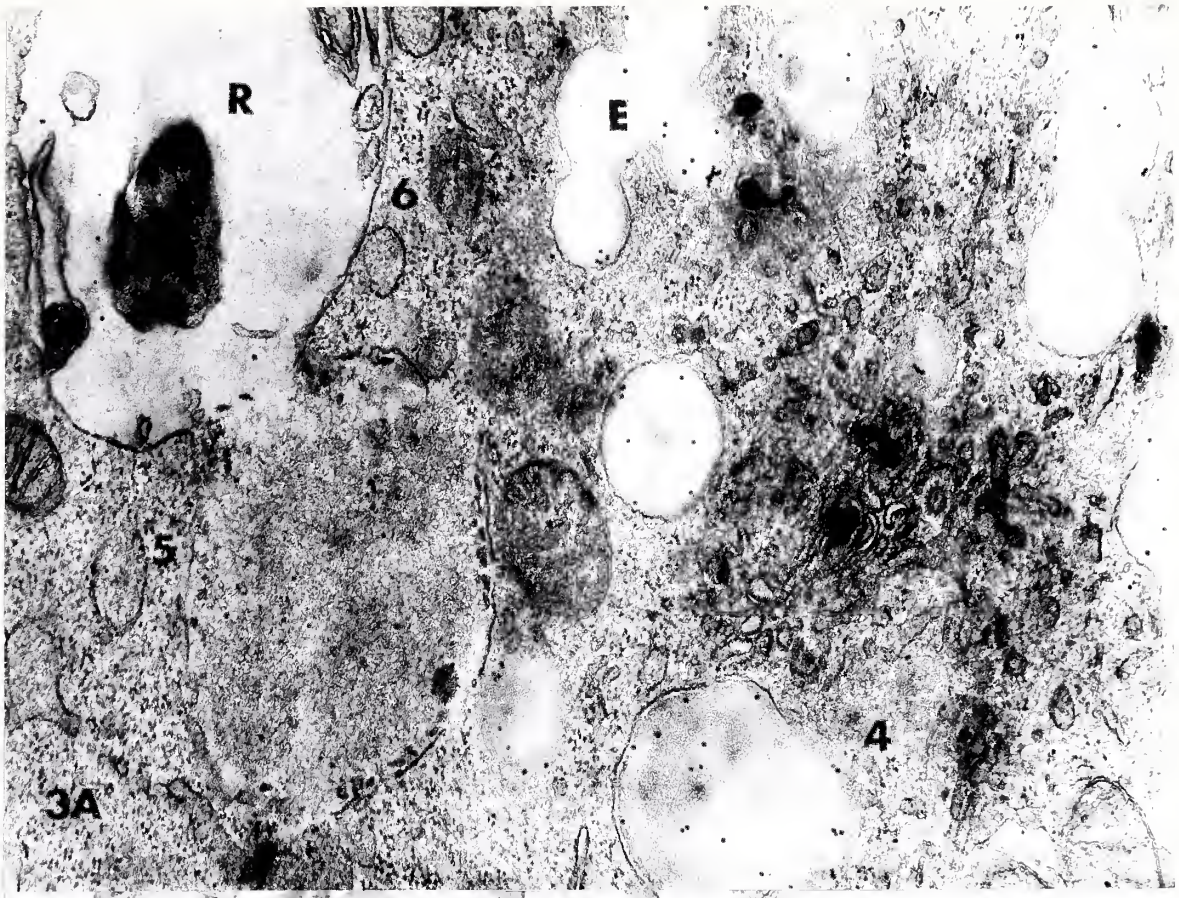
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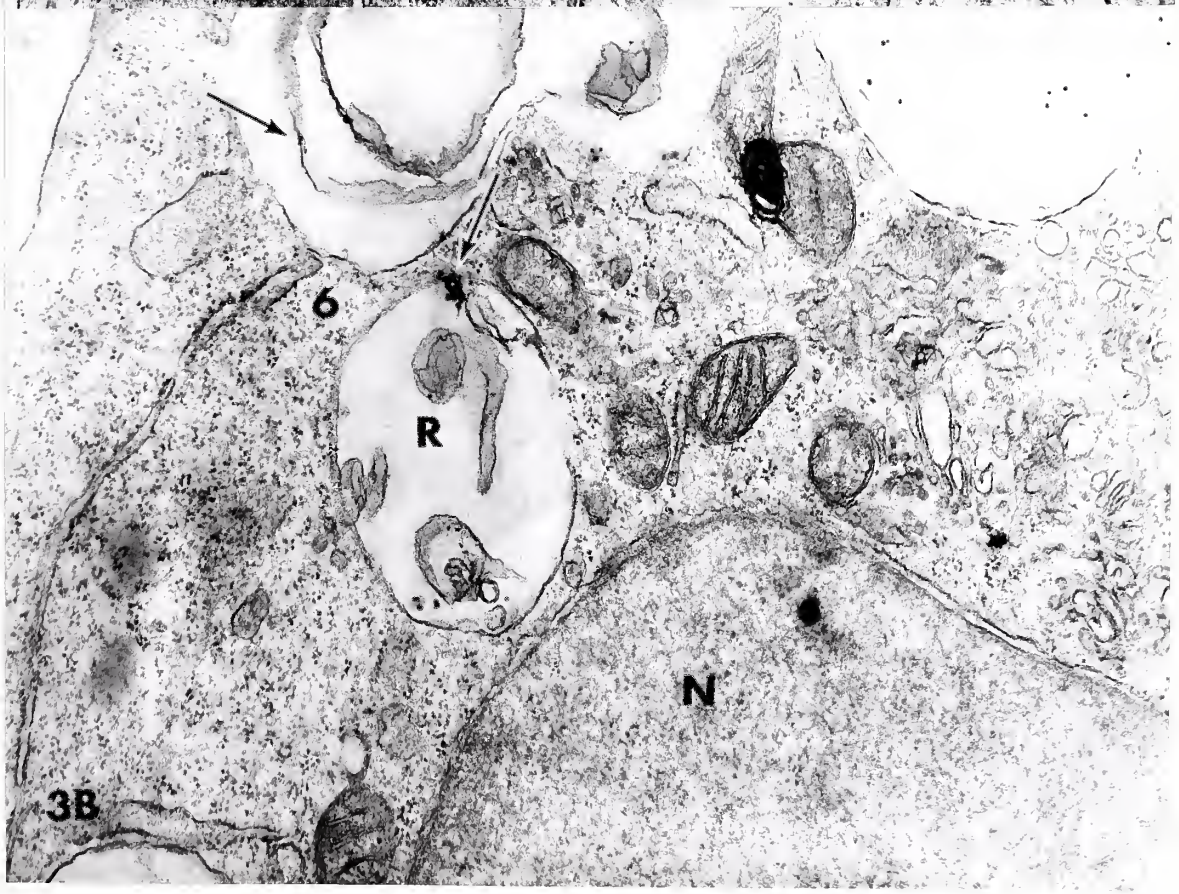
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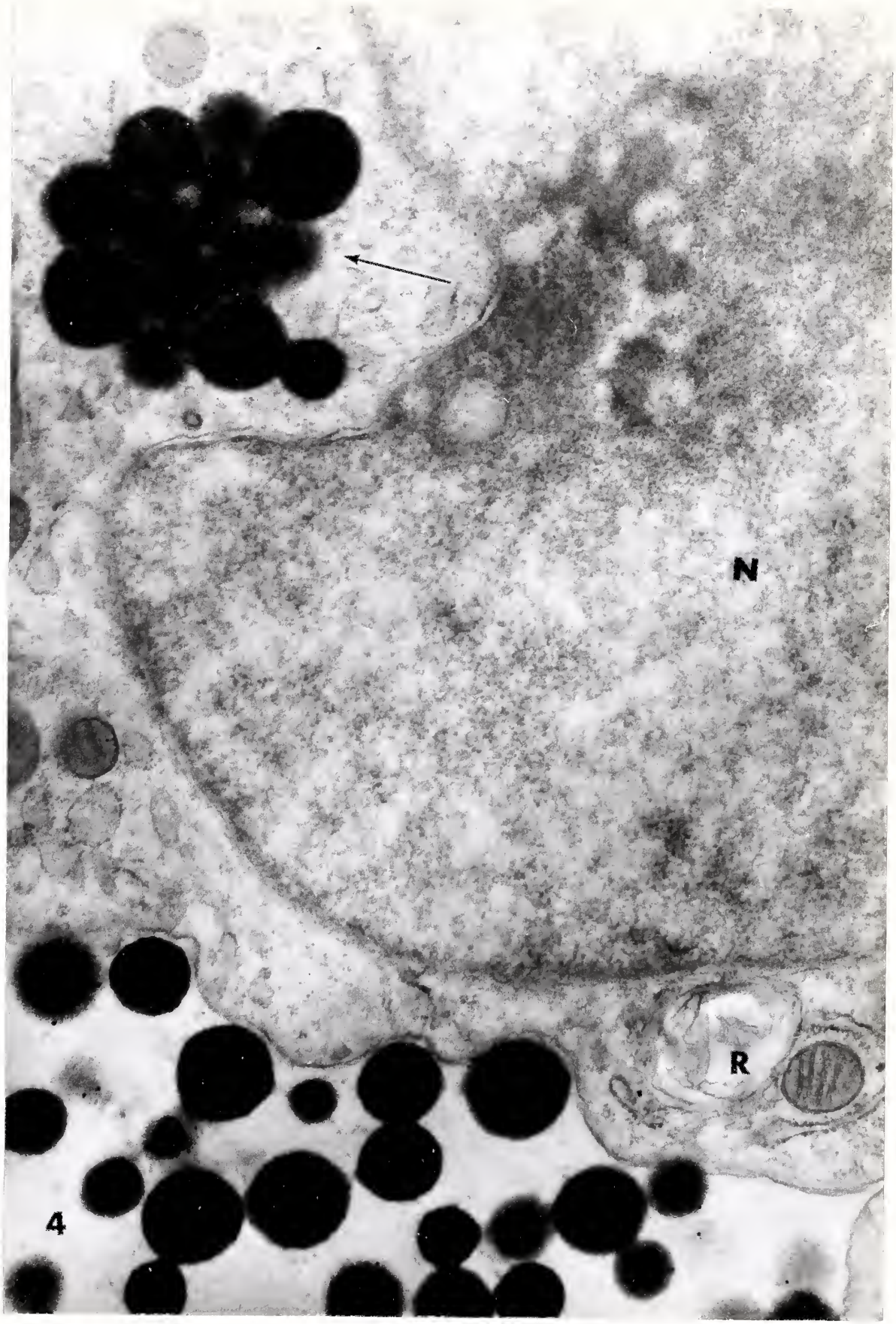
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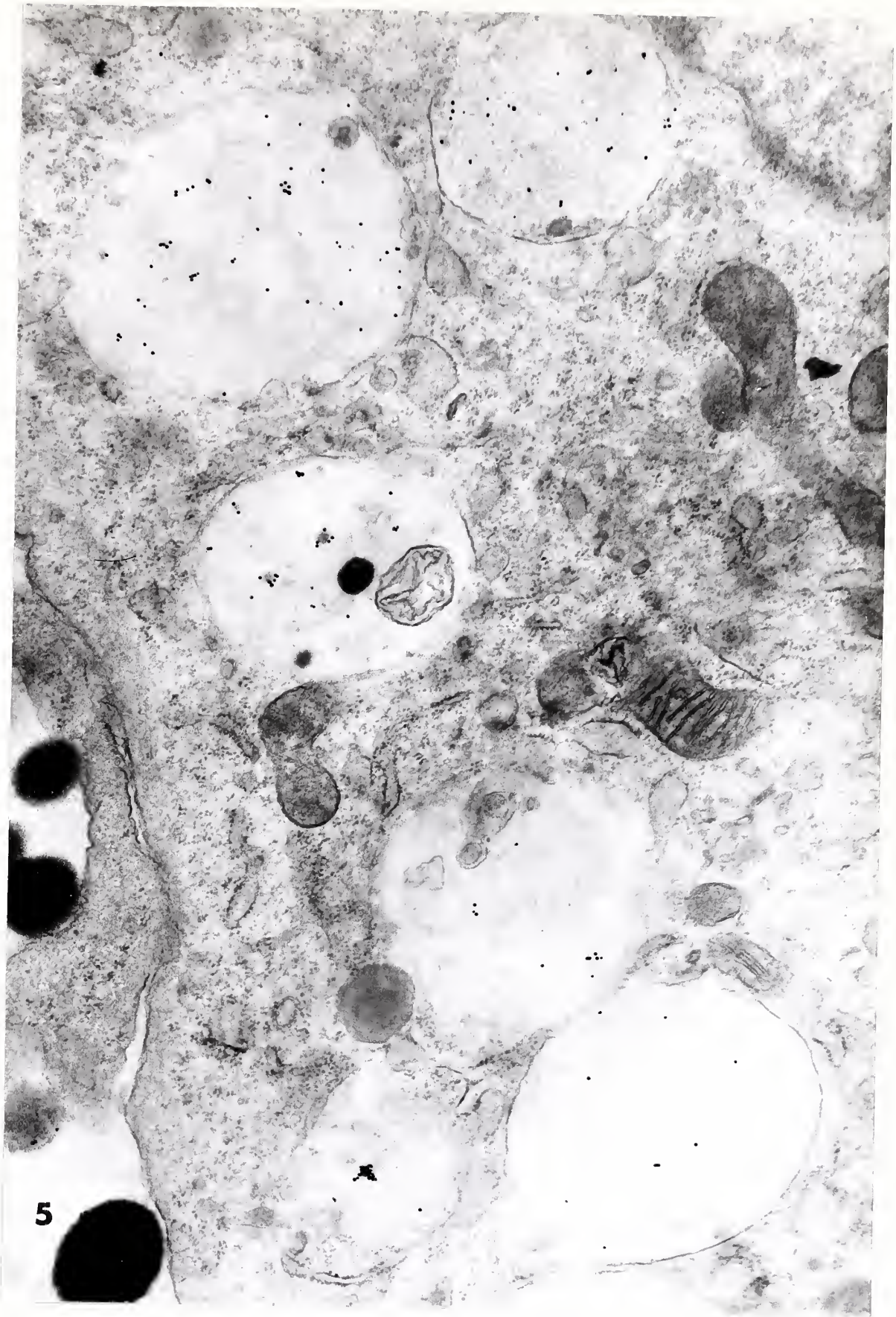

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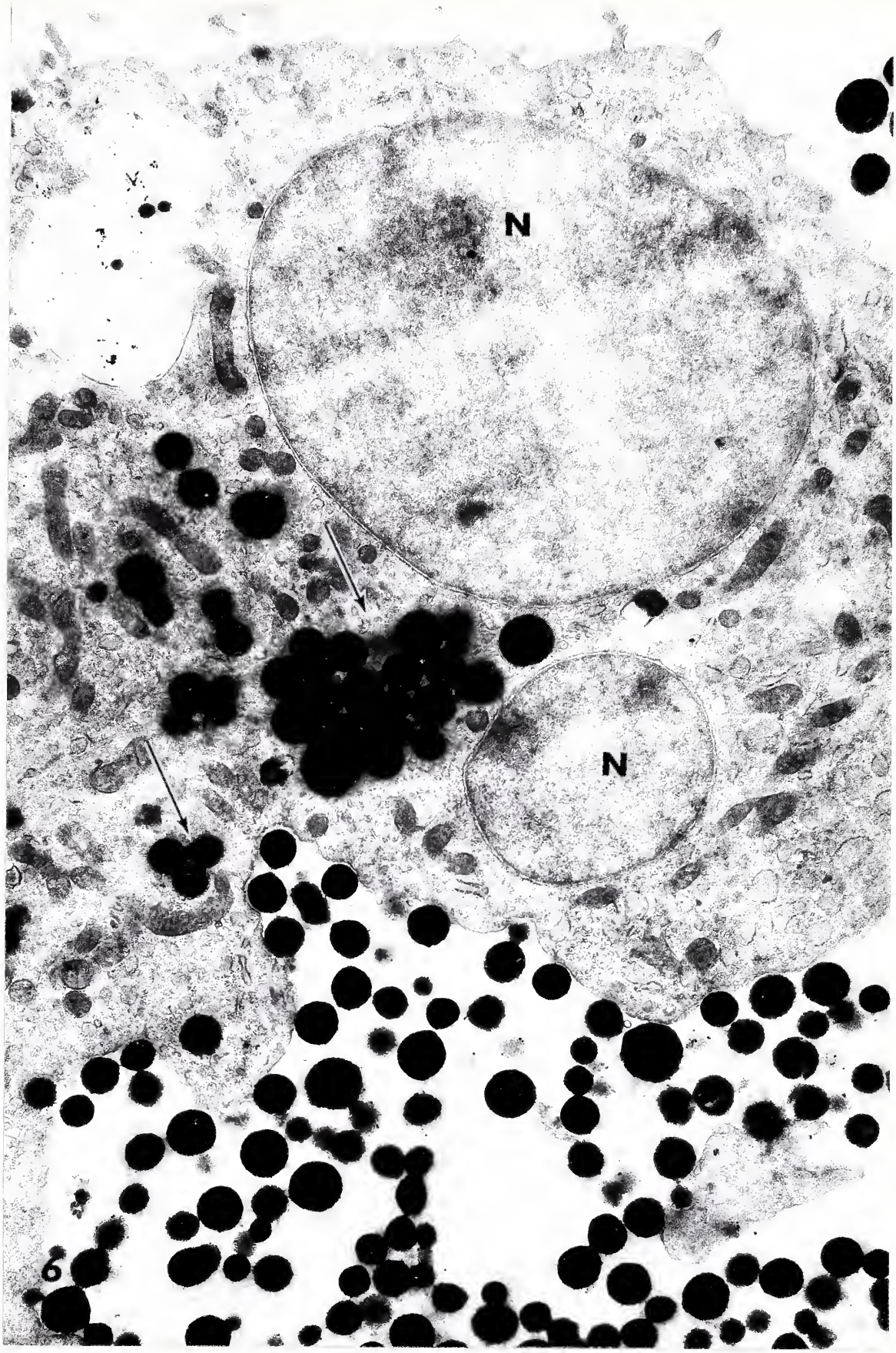
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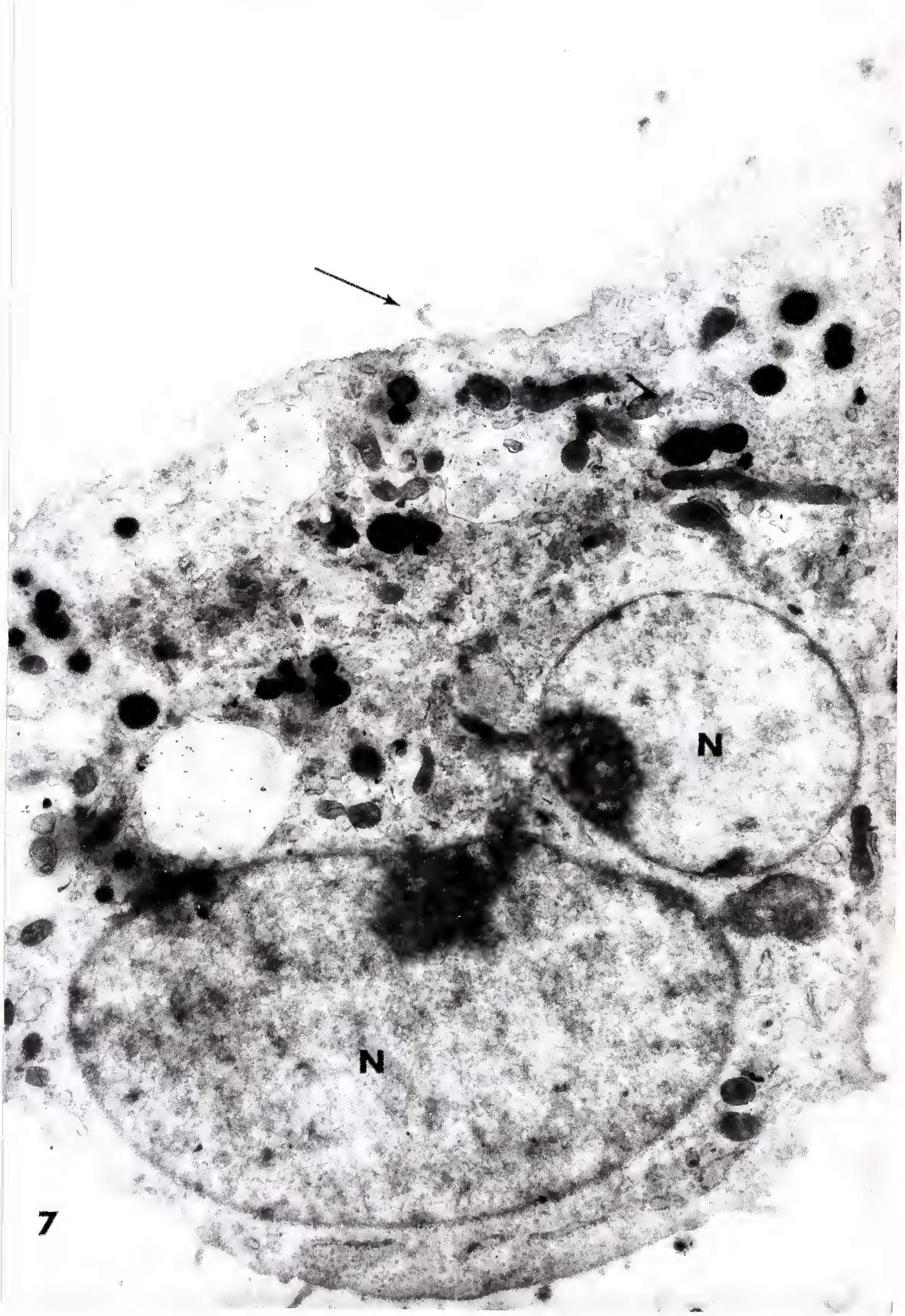

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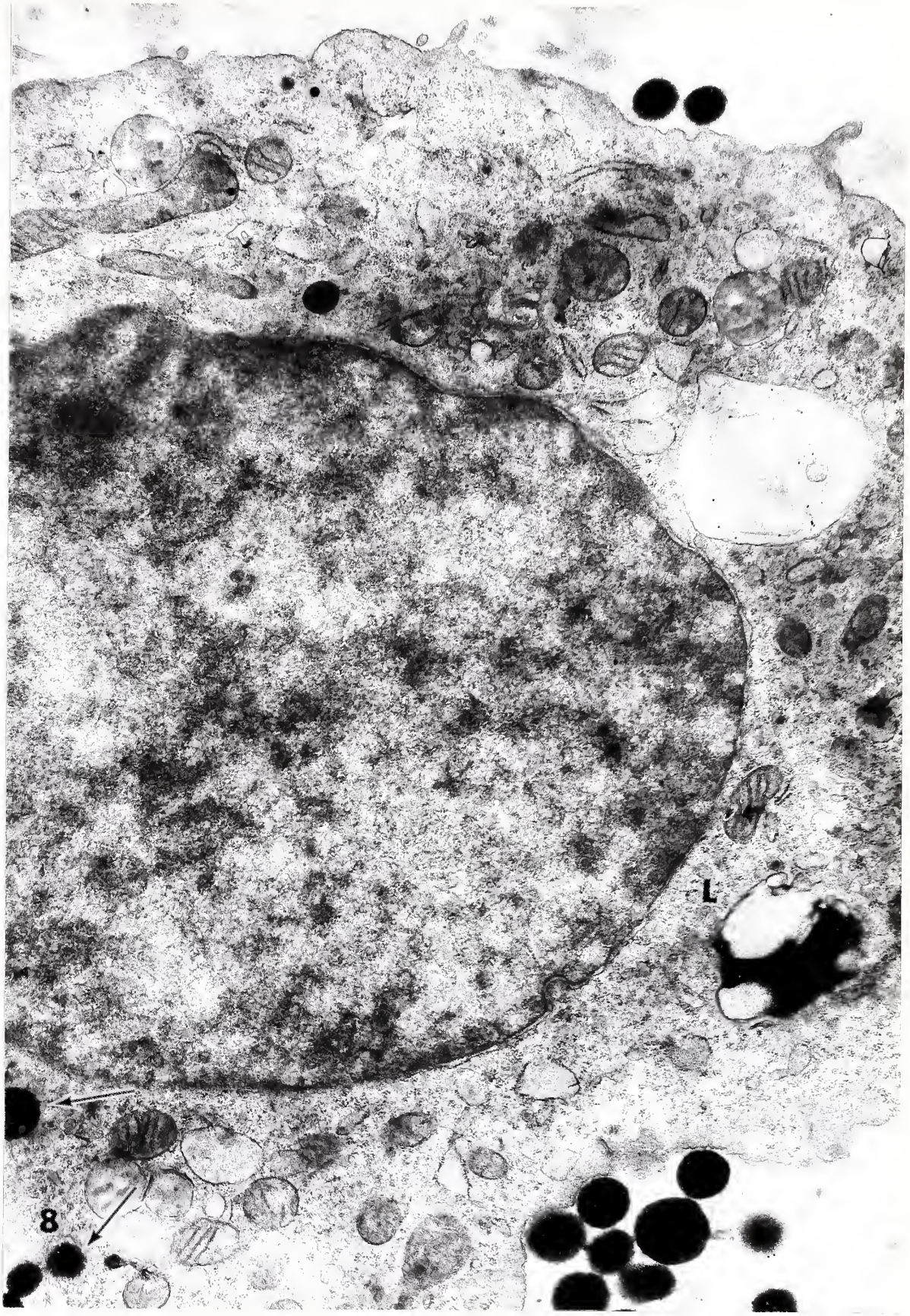
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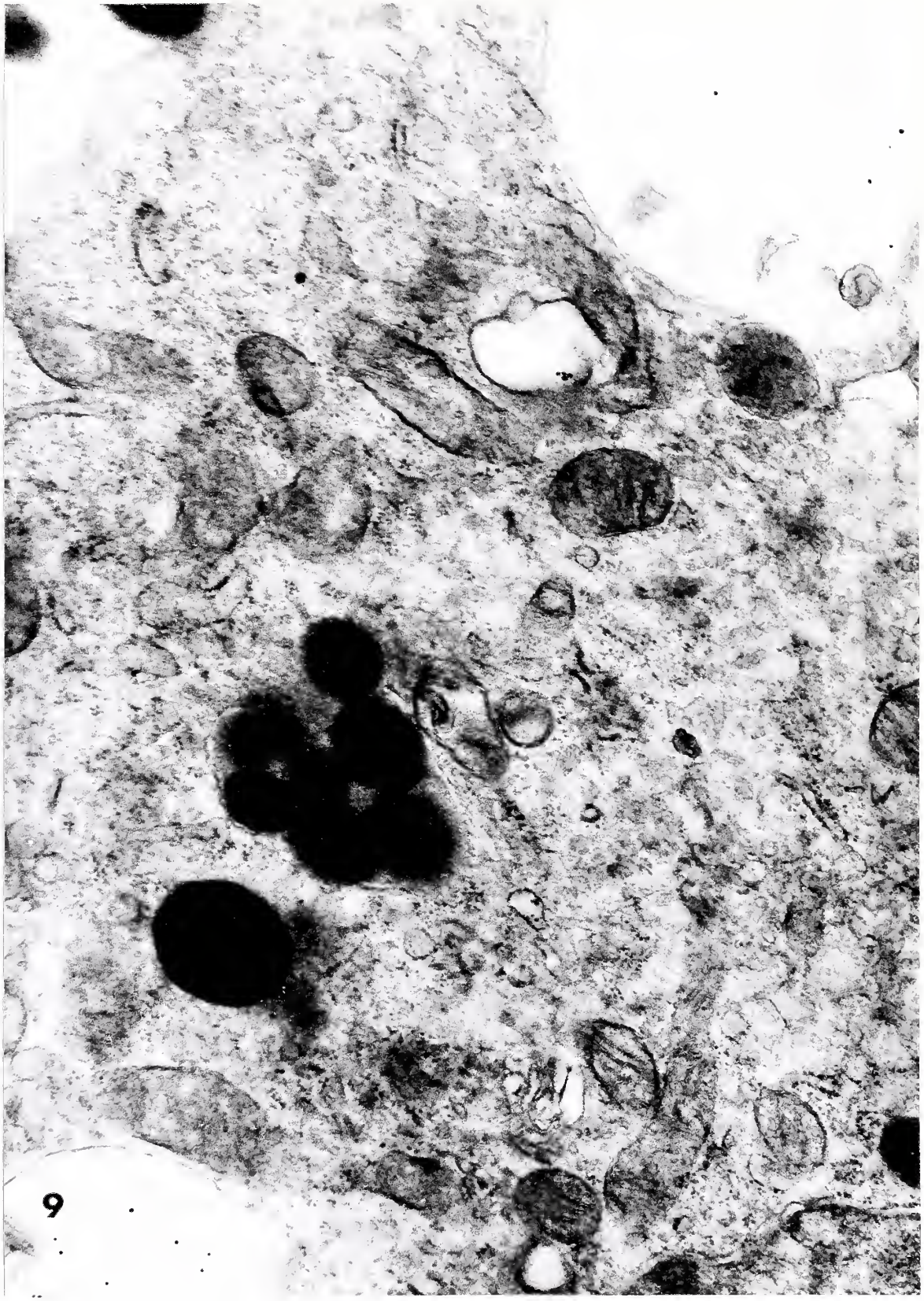

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